My thanks are due to Prof. P. F. Frankland, Dr. G. J. Fowler, and Edward Ardern, M.Sc., for the interest which they have taken in this work and to the Rivers Committee of the Manchester Corporation for permission to publish the results of this investigation carried out in the laboratory of their sewage works at Davyhulme.

The Production of Anthocyanins and Anthocyanidins.

By Arthur Ernest Everest, M.Sc., Lecturer in Chemistry, University College, Reading.

(Communicated by Prof. F. Keeble, F.R.S. Received February 16,—Read March 26, 1914.)

The idea that the anthocyan pigments are closely related to the flavone and flavonol glucosides is by no means new. Attempts to solve the problem of their relationship have come chiefly from botanists, and, as a result of their researches, a number of hypotheses have sprung up around which quite considerable controversy has been centred.

Miss Wheldale* puts forward the suggestion that anthocyan pigments are the oxidation products of colourless or faintly coloured chromogens; and that these chromogens are products of hydrolysis of glucosides present in the tissues of the plant (probably glucosides of flavone or flavonol derivatives). The hydrolysis of the glucoside she considers as essential to the production of the anthocyan pigment. She represents the changes taking place by means of the following equations:—

$$\text{Glucoside} + \text{water} \rightleftarrows \text{chromogen} + \text{sugar}.$$

Then— $\quad$ Oxidation of chromogen $\rightarrow$ anthocyan pigment.

If this hypothesis be accepted, then either the anthocyan so produced will remain a non-glucoside, *i.e.*, it will be an anthocyanidin, or in the presence of sugars the anthocyanidin first formed must unite with sugar to form an anthocyanin (glucoside). Her more recent suggestion that in flavone glucosides all the hydroxyl groups are substituted by sugar molecules, hence partial hydrolysis could produce glucoside anthocyans,† has apparently no foundation upon experimental evidence, most of the flavone and flavonol glucosides containing one or two sugar residues only.

Now, in view of the fact that it has recently been shown that in no case

* 'Camb. Phil. Soc. Proc.,' vol. 15, p. 137 (1909); 'Journ. Genetics,' vol. 1, p. 133 (1911).

† 'Biochem. Journ.,' vol. 7, p. 87 (1913).

could any trace of anthocyanidin be found in any of the plants examined,* there remains but one way of explaining their absence if Miss Wheldale's hypothesis is to be retained; namely, by assuming that the rate of formation of anthocyanin (glucoside) from anthocyanidin and sugar is greater than that of the production of anthocyanidin from chromogen by oxidation, and that these reactions take place under similar conditions.

If this were correct one would expect that by taking the yellow glucoside, hydrolysing, then reducing without removal of sugars, the anthocyanidin produced would combine with the sugar present to form an anthocyanin. This is not the case. Evidence all tends to show that Miss Wheldale's view can no longer be accepted as explaining the available facts.

The reaction found so useful in determining the presence or absence of glucosidal or non-glucosidal anthocyan, and already described by Willstätter and Everest (*loc. cit.*), yields a very ready means of distinction between these two classes of compounds, and has led to important results in the present investigation. It depends upon the facts that anthocyanidins (non-glucosides) are taken quantitatively from aqueous acid solutions, preferably sulphuric acid, by shaking with amyl alcohol, whereas anthocyanins (glucosides) remain quantitatively in the aqueous acid when similarly treated; and, further, that the non-glucoside in amyl alcohol, shaken with sodium acetate solution, remains quantitatively in the amyl alcohol, but on shaking with sodium carbonate solution it is quantitatively carried down into the aqueous layer.

The author has been able to show the production of anthocyanins from yellow glucosides, and that in the cases where hydrolysed solutions were taken only anthocyanidins were produced. That no intermediate formation of anthocyanidins occurred when anthocyanins were obtained was shown by carrying out the formation under amyl alcohol. Where glucoside yellow pigments were used the anthocyanin appeared as usual, but no anthocyanidin passed into the amyl alcohol; when a hydrolysed solution was similarly treated the amyl alcohol rapidly took up all the anthocyanidin as it was produced.

This makes it necessary to abandon the assumption suggested above as the only explanation available if Miss Wheldale's hypothesis is to remain.

A number of papers have been published upon this subject by Keeble, Armstrong, and Jones,† and they conclude that the anthocyan pigments are

* Willstätter and Everest, 'Annalen,' vol. 401, p. 189 (1913).

† 'Roy. Soc. Proc.,' B, vol. 85, p. 214 (1912); B, vol. 86, pp. 308 and 318 (1913); B, vol. 87, p. 113 (1913); and Keeble and Armstrong, 'Journ. Genetics,' vol. 2, part 3, p. 277 (1913).

produced in a manner similar to that propounded by Miss Wheldale, but they part company with that author in regard to the process necessary subsequent to hydrolysis of the glucosides, for they maintain that the oxidation must be preceded by reduction of the non-glucoside flavone or flavonol derivative.

The result of work already published on the pigment of the cornflower,* and consideration of the work of Keeble, Armstrong, and Jones, have led the author to the conclusions (1) that if the anthocyans are produced from the yellow glucosides, then it must be by some interaction in which the glucosides and not the hydrolysed glucosides take part; and (2) that all evidence obtained in dealing with the above-mentioned pigments tends to show that the anthocyan pigments would prove to be, not oxidation, but reduction products of the yellow glucosides.

That luteolin and morin give red pigments on reduction in acid alcoholic solution by means of sodium amalgam has been known for many years.† Quite recently Watson‡ has obtained a red pigment from quercetin by the same means, and, lastly, since the present work was completed, the author's attention has been drawn to a paper by R. Combes,§ who describes the production by the same means from the yellow pigment of the green leaves of *Ampelopsis hederacea* of a pigment which he shows to be identical with the red pigment (anthocyan) which he obtained from the red leaves of the same plant. He does not, however, give definite information whether his pigments are anthocyanins or anthocyanidins. The author is able to confirm the production of anthocyan pigments by reduction of flavone or flavonol derivatives, and to explain the observations of Keeble, Armstrong, and Jones. Being one of the most commonly occurring of the flavonol class, and readily obtained, quercitrin (Kahlbaum) was taken for the starting point.

Quercitrin by reduction with zinc (fine granulated) and dilute acids (2N HCl) or by electrolytic reduction, even cold, gave only anthocyanidin.

The pigment production took place equally well when the aqueous acid was covered with a layer of ligroin, so precluding all possibility of oxidation by the air following reduction. As, however, no anthocyanin could be obtained from quercitrin (this is a monoglucoside from bark, not a flower pigment, at least some flowers are known to contain a diglucoside of quercitrin, *e.g.*, viola contains viola quercitrin)—a result which at first appeared to support Miss Wheldale's hydrolysis hypothesis—the author

* Willstätter and Everest, *loc. cit.*

† *Cf.* Rupe, 'Die Chemie der natürlichen Farbstoffe,' vol. 1, pp. 77 and 85.

‡ 'Chem. Soc. Proc.,' 1913, p. 349.

§ 'Compt. Rend.,' vol. 157, p. 1002 (1913); 'Chem. Zentr.,' 1914, p. 158.

decided to test the pigments obtained by direct extraction of various flowers, in particular with a view to producing anthocyanins from the yellow glucosides present in them.

Having already shown that oxidation after reduction was not necessary for the production of anthocyan pigments—and this was confirmed in every case where reduction under ligroin was carried out—particular attention was given to proving that anthocyanins could be produced directly from yellow glucosides without intermediate formation of anthocyanidins, and in this the author was successful.

The yellow wallflower, yellow daffodil, white narcissus, yellow or white tulip, white primula (*obconica*), yellow crocus, yellow jasmin, yellow primrose (the presence of yellow pigments in the white flowers was shown by action of dilute ammonia, when, the plant acids being neutralised, the yellow colour appears), and even lemon peel, all yielded by reduction alone red pigments, and, indeed, pigments which upon investigation proved to be in every case an anthocyanin, no trace of anthocyanidin being produced when the reductions were carried out in the cold. No oxidation after reduction was necessary for the production of the anthocyanin pigment, provided that in one or two instances care was taken not to carry the reduction too far.

Reduction was carried out by zinc (fine granulated) in *ca.* 2N aqueous acids, and also by electrolysis in 2N sulphuric acid, using lead electrodes (lead has been found to yield salts with anthocyanins, which, however, are decomposed by acids; lead salts have no harmful effect upon anthocyanins).

At first some difficulty was experienced in explaining the observations of Keeble, Armstrong, and Jones* that in the case of yellow wallflower, yellow daffodil, yellow crocus, cream polyanthus, and Chinese primrose oxidation was necessary after reduction in order to obtain a red pigment. A ready explanation was, however, forthcoming when the case of the yellow tulip was examined, for here, when reduction was rapid, there appeared but a transient pink, passing rapidly to a colourless solution, which, however, on addition of hydrogen peroxide immediately developed a red colour. Slow reduction, however, by zinc (very small quantities) and HCl or, much better, slow electrical reduction gave readily the red pigment, and this proved to be as in the other cases an anthocyanin. The red solution on stronger reduction passed to a colourless one, from which the anthocyanin was again produced by the addition of hydrogen peroxide.

It has been found that in each case excessive reduction produced to a greater or less extent the above result, and this clearly explains the results of Keeble, Armstrong, and Jones (*loc. cit.*).

* 'Roy. Soc. Proc.,' B, vol. 87, p. 113 (1913).

On a previous occasion attempts to reduce cyanin (the pigment of the cornflower*) to a colourless compound which could be re-oxidised to the pigment had failed. Powdered zinc and acetic acid were used, hot—the pigment was decolorised, but the red colour was not reproduced on addition of hydrogen peroxide. Despite this fact, the author, for comparison, treated a small quantity of cyanin chloride in 2N hydrochloric acid with much finely granulated zinc, so that vigorous evolution of hydrogen ensued. The reaction was carried out in the cold, and, as in the cases mentioned above, decolorisation rapidly set in, but on decanting the decolorised solution and adding hydrogen peroxide the colour reappeared. The glucoside was not hydrolysed by this process.

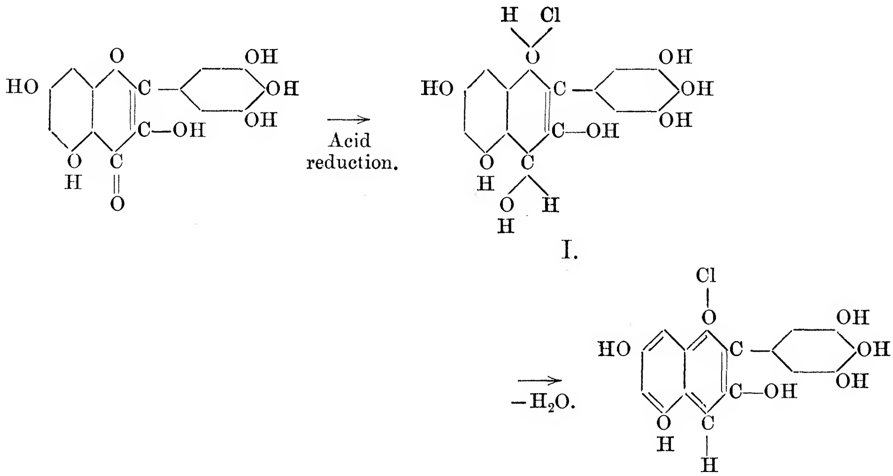
In every case, also with cyanin chloride, when treated with hydrogen peroxide in the cold, the red acid solution of the anthocyanin passed to a yellow, then became colourless. It would thus seem that the balance of reducing powers present in an anthocyan-containing flower must be very finely adjusted, for it appears necessary that the reducing body present should be powerful enough to reduce as far as the anthocyanin stage, but not powerful enough to take the pigment further to the colourless condition.

It has been placed beyond doubt that the change from yellow to red may be accomplished by reduction alone, thus confirming the results of Combes, and, still further, that the change from glucoside flavone or flavonol to anthocyanin (glucoside) takes place quite readily without hydrolysis, and that all hypotheses which require a hydrolysis of the glucoside before formation of red pigments can, in the light of the evidence of Willstätter and Everest, that the anthocyanidins do not exist in plants, and the further evidence now brought forward, that flavone or flavonol glucosides readily yield anthocyanins without intermediate formation of anthocyanidins, be discarded as unnecessary.

Whether all the yellow glucosides of the flavone and flavonol series are capable of producing corresponding anthocyanins remains to be proved by future work. The author failed to observe such formation in the case of *Primula sinensis* (Giant white), mimosa, and white hyacinth. (Whites tested with ammonia gave yellow.)

Whether the red pigments described above should be considered as mere hydroflavone derivatives as I, or as some such anhydro-compound of them, II, remains to be proved, but the author considers that the evidence at present available favours some such form as II, where the change has caused the production of a quinonoid structure, as follows :—

* Willstätter and Everest, *loc. cit.*



I. II.

In this connection an examination of the properties of cyanin chloride and cyanidin chloride* is of interest. Cyanidin chloride when heated for a short time in dilute alcohol to *ca.* 80° becomes decolorised—the decolorised substance has properties resembling those of a yellow flavonol pigment, soluble in ether, colourless in acid solution, extracted from it by ether; yellow in alkaline solution, and alkalis withdraw it from its solution in ether. The decolorised cyanidin chloride, however, on boiling with acids, returns to the red form. It is possible that these changes may be represented by the change from I to II above being a reversible reaction. The fact that a decolorised solution of cyanidin chloride on concentration regains its colour also harmonises with the above.

Quite similar properties are observed in the case of cyanin chloride, save that heat is not required for decolorisation, nor for return of the red pigment on acidification. Extraction of the decolorised solution with ether was not tried by Willstätter and Everest, but alkalis on the decolorised solution gave a yellow coloration. Most probably the general character of the groups in the molecule would have their effect on the readiness with which this change took place, and hence this decolorisation. Such a change might perhaps explain the observation of Keeble, Armstrong, and Jones (*loc. cit.*), that in the case of polyanthus mere boiling with acid was sufficient to produce the red pigment.

As he learns that recent developments in the work of Prof. Willstätter and his collaborators have caused them to commence a series of investigations dealing with the relation between the yellow pigments and the anthocyans the author proposes to discontinue these investigations for the present.

* Willstätter and Everest, *loc. cit.*

Experimental.

Quercitrin.—Reduction carried out in 2N HCl by zinc (fine granulated).

(1) Hot, yielded rapidly a red solution.

(2) Cold, gave red coloration but very slowly.

(3) Cold, alcoholic HCl and Na Hg: rapidly gave red pigment.

(4) Cold, electrolysis in 2N sulphuric acid, lead electrodes: very slow production of red.

(5) The best method, however, of obtaining the red pigment from quercitrin is by the action of magnesium (ribbon or turnings) on a solution of the substance in a mixture of 5 vols. absolute alcohol and 1 vol. concentrated hydrochloric acid. Not only does this go very readily, but the acidity of the solution—so essential in working with these compounds—is preserved as the magnesium practically ceases to react before the solution becomes neutral. This method was of no value when working with crude plant extracts, as alcoholic extracts contained so much extraneous matter that the results were masked.

In every case pigment, when shaken with amyl alcohol, went quantitatively into the alcohol, solution red with tinge of violet; shaken with sodium acetate solution, pigment remained quantitatively in alcohol, turned violet; shaken with sodium carbonate solution, pigment descended quantitatively into aqueous layer with green colour; prepared by method (5) and purified from remaining quercitrin, solution gave blue solution in sodium carbonate. The red pigment was not extracted from aqueous acid by any other organic solvent.

Yellow Wallflower.—Petals from a few flowers crushed in mortar with fine sand and cold 2N HCl, then filtered, gave a faintly yellow extract which with ammonia became deep yellow. To one portion of acid extract a small quantity of zinc was added, whilst a second portion was kept for comparison, to show that no red developed without the reduction. In a few minutes the portion containing zinc became pink and the colour rapidly deepened to red. Blank portion remained unchanged. The pigment produced by reduction remained quantitatively in the aqueous layer when shaken with amyl alcohol, but if the layer was separated, then boiled to hydrolyse the pigment, and then again shaken with amyl alcohol, the red pigment then went quantitatively into the alcoholic layer with production of a red solution. This reacted in every way as an anthocyanidin. Electrolysis also produced the anthocyanin and only that, no trace of anthocyanidin was produced by reduction in the cold.

Reduction in hot solution produced anthocyanidin and no anthocyanin.

Reduction under ligroin produced the same results as without protection from air.

Primula obconica (white).—Petals gave clear yellow on treatment with ammonia, no pink with acids. Extract made as above, almost colourless. Reduction with zinc in cold 2N HCl gave good red pigment. The reduction went equally well under ligroin, and in both cases the pigment produced was quantitatively anthocyanin, and could be hydrolysed quantitatively into anthocyanidin.

Primula sinensis (Giant white).—Petals gave clear yellow with ammonia, no pink with acids. All attempts to obtain a red pigment failed.

Tulip (yellow).—Extract prepared as above in 2N HCl. With much zinc a faint passing pink colour appears, then solution becomes decolorised, hydrogen peroxide added to the decanted solution causes appearance of red colour. Exposure to air has same effect. The red produced is an anthocyanin readily hydrolysable to an anthocyanidin.

When acid extract was treated with small quantities of zinc the pink colour soon appeared and deepened. If not taken too far hydrogen peroxide caused no change.

Electrolysis of cold extract in 2N sulphuric acid, lead electrodes, readily gave the red pigment which, as in the preceding cases, proved to be entirely anthocyanin.

Tulip (white).—Petals with ammonia gave clear yellow. Exactly similar results were obtained as for the above yellow tulip. Both in the case of yellow and white tulips the reduction went on equally well under ligroin.

When the extract from the white tulip was boiled to hydrolyse the glucoside contained, then cooled and reduced in the cold, a red pigment was readily obtained, but it was entirely an anthocyanidin.

Daffodil (yellow).—Extract as before, zinc in 2N HCl gave red pigment easily. Electrolysis in 2N sulphuric acid gave same result. In both cases cold reduction gave only an anthocyanin. Reduction went on to red pigment equally well under ligroin.

Narcissus (small white).—Petals with ammonia gave clear yellow. Reduction with zinc in 2N HCl gave only anthocyanin.

Mimosa.—All attempts to get red pigment failed.

Hyacinth (white).—Petals gave yellow with ammonia, but all attempts to obtain red pigment failed.

Crocus (yellow).—Extract as above gave, by zinc in 2N HCl, or by electrolysis in 2N sulphuric acid, red pigment quite readily, in both cases cold reduction yielded only anthocyanin.

Jasmin (yellow).—Gave anthocyanin only, more readily by means of zinc and 2N HCl than by electrolysis.

Primrose (yellow).—Easily produced anthocyanin by either method, even in fairly warm HCl with zinc only anthocyanin was produced. The glucoside produced in this case seemed to be more stable to hydrolysis than in the majority of cases.

Lemon Peel.—Extract in HCl, in presence of the peel, reduced with zinc gave only anthocyanin.

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*Variations in the Growth of Adult Mammalian Tissue in Autogenous and Homogenous Plasma.**

By Albert J. Walton, M.S., F.R.C.S., B.Sc.

(Communicated by Prof. W. Bulloch, F.R.S. Received February 18,—Read March 26, 1914.)

(From the Bacteriological Laboratory of the London Hospital.)

[Plates 19 and 20.]

In 1910 Carrel commenced his researches on the growth of tissues outside the body. In 1907 Harrison had succeeded in growing the embryonic tissues of the frog, using coagulable lymph as a medium. In 1910 Harrison and Burrows improved this method and successfully cultivated the tissues of mammalian embryos. Carrel has so modified the technique that the method is now applicable to the study of the growth of all mammalian tissues. He used as a medium the plasma of the animal either in its natural state or modified by the addition of various substances. Since then, he and his collaborators have published a large number of papers, and by their work it has been fully established that tissues of animals, whether embryonic or adult, grow well *in vitro*; that by changing the medium and so removing the catabolic substances life can be greatly prolonged—tissues have been kept alive and growing for periods considerably longer than a year; and that the growth of the tissues can be greatly modified by the addition of various substances to, or otherwise altering the composition of, the plasmatic medium.

* Throughout this paper the term "autogenous" is used to indicate plasma obtained from the same animal as the tissue, "homogenous" to indicate that obtained from another animal of the same species.